# PATENT ABSTRACTS OF JAPAN

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(71)Applicant : RES DEV CORP OF JAPAN
TSUNODA HIDEO
(72)Inventor : TSUNODA HIDEO

## (54) METHOD FOR INTRODUCING BIOSUBSTANCE TO CELL USING MAGNETIC FINE PARTICLE AND SELECTIVE CONCENTRATION AND SEPARATION OF CELL BY MAGNETISM

(57)Abstract:
PURPOSE: To enable magnetic concentration and separation of a cell containing a biosubstance such as gene introduced into the cell by shooting fine magnetic particles supporting immobilized biosubstance into a cell at a high speed.
CONSTITUTION: A biosubstance is immobilized on fine magnetic particles and the particles are shot into a cell at a high speed. The cell containing the introduced fine magnetic particles can be selectively and specifically concentrated or separated by a magnetic means. The method is useful for the screening of a drug resistant cell, etc.

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CLAIMS

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[Claim(s)]
[Claim 1] The intracellular introductory approach of the biological substance characterized by firing into intracellular the magnetic particle which fixed the biological substance at high speed.
[Claim 2] Alternative concentration and the separation method of the cell characterized by condensing or separating alternatively the cell into which the magnetic particle was introduced by the introductory approach according to claim 1 with the MAG.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Industrial Application] This invention relates to the approach the MAG condenses or separates alternatively the cell introduced by the approach and this approach of introducing biological substances, such as a gene and an enzyme, to a cell by the magnetic particle.
[0002]
[Description of the Prior Art] About the magnetic particle, P.J.Robinson and others (Biotechnol.Bioeng., 15, 603-606 (1973)) is performing the research report using a magnetic particle as immobilization support of an enzyme as immobilization support of the enzyme for bioreactors paying attention to the point with the easy recovery actuation. By this report, alpha-chymotrypsin or the beta-galactosidase is fixed in an iron-oxide particle or cellulose-iron-oxide complex, it uses for a complete-mixing mold bioreactor, and it is shown that it can condense magnetically and easily and that magnetic support can be separated.
[0003] Moreover, the iron ultrafine particle (the minor axis of about 30nm, major axis of about 500nm) which has magnetism is encapsulated with synthetic macromolecule, and the support of the shape of an ultrafine particle which fixed the enzyme which can be guided magnetically is made by fixing an antibody and an enzyme in the outer surface (editing besides "ultrafine particle creation technology" wood Chikara, the Mita Press issue, and 231-235 page). Here, as a result of making it react with a small bioreactor using the magnetic ultrafine particle support which fixed glucose oxidase as a model enzyme, the reaction rate per unit volume is high, and it is shown that the support of an ultrafine particle can be condensed and collected easily magnetically.
[0004] Furthermore, it is a particle of a macromolecule which distributed the magnetic particle. Import sale of the DYNABEADS (trademark) is carried out from Japanese Dynal. This product be the particle (a diameter 2.8 and 4.5 mum ) which made polystyrene beads distribute ferric oxide (a ferrite , Fe 2O3 ) for the purpose of magnetic separation of the protein of an antibody and others , a nucleic acid , etc. , and when the coat of the antibody be carry out to this front face or the bead which combined a certain kind of protein or a nucleotide etc. use a magnet together , separation and purification of a cell can be promptly perform by simple actuation .
[0005] However, it fires, and as a particle of business, the thing using the complex of a magnetic particle and a macromolecule as support has a low consistency, and since the impulse force of a collision is weak, it is unsuitable [ a thing ] among the biological substance fixed magnetism particles mentioned above. Moreover, particle size is not small enough compared with the magnitude of a label-cell. therefore, the vegetable intracellular installation which has a cell wall -- impossible -- **** -- the precedent does not exist. Moreover, although it may be theoretically possible in the microinjection method using the capillary of glass, it is difficult to introduce into many cells at once.
[0006] Moreover, particle support is made for a gene to adhere to the U.S. Pat. No. 5,100,792 specification by J.C.Sanford and others, it introduces into intracellular at high speed, and the method (party Kurgan law) of making the function discover is indicated, and in order to enlarge collision energy which a particle has here, gold and a tungsten are mainly used. It is the diameter of 4 micrometers which is concretely used in the example in this U.S. Pat. No. 5,100,792

specification. It is a tungsten particle and they are about 10nm thru/or several micrometers about the particle size of a particle in a detailed description. It is indicated and the ferrite crystal of high density (about ten to 20 g/cm3), gold, a tungsten, other metal particles, the glass of a low consistency (1 - 2 g/cm3), polystyrene, and a latex bead are indicated.
[0007]
[Problem(s) to be Solved by the Invention] This invention aims at making intracellular installation and its intracellular functional manifestation of a gene etc. of a biological substance perform using the particle which has the magnetism of a physical property. Moreover, this invention aims at condensing alternatively and specifically and separating only the cell which the magnetic particle introduced into intracellular used magnetically the point which can be guided, for example, the gene discovered or the physiological function discovered, and a cell lump and an organ.
[0008] Plastics bead containing a conventional magnetic particle and a conventional magnetic particle (compounded magnetic microsphere) In all cases, such as an application, the magnetic particle was out of the cell, and it is impossible to current to put in a magnetic particle inside [ which has a hard cell wall like especially vegetation ] a cell, and it did not prove globally to this invention.
[0009]
[Means for Solving the Problem] It is the intracellular introductory approach of the biological substance characterized by this application the first invention firing into intracellular the magnetic particle which fixed the biological substance at high speed, and this application the second invention is alternative concentration and the separation method of the cell characterized by condensing or separating alternatively the cell into which the magnetic particle was introduced by this introductory approach with the MAG.
[0010] As a magnetic property in the magnetic particle used for this invention, if it is the magnetic substance, what kind of thing may be used, for example, ferromagnetism, a paramagnetism, and the superparamagnetism matter are used. As the quality of the material of this magnetic particle, for example A metal, a metallic oxide, nonmetal-metal complex, Although nature with ceramic complex and the above-mentioned matter, the organic magnetic substance containing a synthetic organic compound and its complex, the fluorescence matter, the optical responsibility matter, the optical magnetic functionality matter, the electron transport matter and the electrochemical functionality matter, the quantum-mechanical functionality matter, and semi-conducting material are mentioned From the passing speed by MAG, such as a cell which contains the particle in case magnetic induction separation and concentration are performed, being large The magnetic-substance particle which contains the compound particle of ferromagnetic elements, such as a ferromagnetic compound, for example, magnetite, other iron, cobalt, and nickel, and chromium, manganese, aluminum, an yttrium, a tellurium, a tungsten, titanium, etc. especially is desirable. Among these ferromagnetic compounds, toxicity is not shown and magnetite is [ in / to a living body / a stable point ] especially desirable.
[0011] As a particle size of this magnetic particle, a maximum grain size is one fifth of object cells. It is possible from grade to the size of an ultrafine particle, and is the mean particle diameter of 5nm - 100nm. An ultrafine particle or its floc can be used and it is the mean particle diameter of 10nm - several micrometers preferably. A magnetic particle is used. the consistency of this magnetic particle -- usually -- 1 - 21 g/cm3 -- desirable -- 3 - 8 g/cm3 it is . Therefore, magnetite (consistency : about 5g/cm 3), hematite (consistency: about 5 g/cm3), A cobalt ferric acid ghost (consistency : about 3g/cm 3), a barium ferrite (consistency : about 5g/cm 3), In addition, a consistency is 3 8g/cm. The carbon steel which is extent, tungsten steel, and KS steel, A consistency is 4 - 5 g/cm3. It is desirable to use the particle of the iron oxide which is extent, zinc, magnesium, manganese, or an iron compound (Ferroxcube) ingredient with nickel, and the particle of a rare earth cobalt magnet (consistency: about 8 g/cm3).
[0012] As for the gestalt of this magnetic particle, what has an angle in a cell wall or a cell membrane in a non-globular form in order to make it easy to break, penetrating and is desirable, and when compared with the same cross section, to a cell wall or a cell membrane, penetrating and since it is easy to overcome, moment of inertia is greatly needlelike, and a rod-like thing has

a cross section more desirable than a spherical particle. In this invention, the biological substance which the above-mentioned magnetic particle is made to fix means physiological active substances, such as an animal, vegetation, protozoa, and a microorganism, an organella, a living thing particle, etc., for example, a gene, an enzyme, an antibody, protein, a pheromone, allomone, a mitochondrion, a virus, etc. are mentioned.
[0013] In this invention, it says that immobilization mainly holds a biological substance to the particle with physical adsorption, its allogene object chemical affinity, etc. at a magnetic particle. The immobilization to the above-mentioned magnetic particle of the top Norio object matter can mix the solution of for example, this magnetic particle, and the buffer solution of a biological substance, and can be performed by carrying at the tip of a projectile and being air-dry.
[0014] As a target cell in this invention, it is an animal cell. (a human cell is included) A plant cell, other living thing cells, an organ, an organization, etc. are mentioned. "it firing at high speed" in this invention — the initial velocity of a particle — per second 50 — it is about -400m — saying — for example, a U.S. Pat. No. 5,100,792 specification and Japanese Patent Application No. 4-25626 party Kurgan given in a number specification etc. — it can carry out by law.
[0015] The MAG can condense or separate alternatively the cell into which the magnetic particle was introduced as mentioned above. This concentration or separation can be performed as follows, for example. For example, the solution which distributed the cell is moved to transparence containers, such as a test tube, a magnet is stuck on the side attachment wall, and a cell is condensed. Then, only the cell containing a magnetic particle is separated by removing other solution parts with a pipet etc. This actuation is repeated several times if needed.
[0016] this invention be the increase in efficiency; physiology effectiveness of transgenics, recombination, and training of recombinant. ( vegetation, animal) drugs, such as installation and magnetic induction of the exist matter, and a preferential segregation; anticancer agent; bioreactor using a gene recombination cell ( for [ for analysis ] - industrial production) sensing of; magnetic gene recombination; intracellular information be applicable to all the technique of a magnetic means and others recognize from the outside by chemical, biochemical, and the approach combined through the physical property and the approach.
[0017]
[Example] Hereafter, although an example explains this invention still more concretely, the range of this invention is not limited to the following examples.
(Example 1) as installation of the gene by magnetite, and a manifestation magnetism particle — magnetite (mean particle diameter 0.3 micrometers and consistency 5.2 g/cm3) (refer to drawing 1 ) using — party Kurgan — installation and its manifestation of a gene were performed using the particle launcher (Japanese-Patent-Application-No. 4-25626 equipment given in a number specification is used) by law.
[0018] In a gene, it is plasmid DNA and pBI221 (made in [ Clonetec ] the U.S.). It used. This plasmid is the beta-glucuronidase. (GUS) Having a gene, this is a cauliflower mosaic virus. (CaMV) 35S promotor (pCaMV35S) and nopaline synthesis enzyme gene (NOS) terminator (pAnos) It connects in between.
[0019] Adhesion actuation to coating and the plastics projectile to a magnetite particle of plasmid DNA was performed as follows. 50mg magnetite particle The solution which ethanol 1ml is made to have suspended 100% is made, and it is 1-2microl about this solution, TE buffer solution (DNA concentration between 200 - 500ng/mul) of plasmid DNA, and the mixed solution optimum dose **** and after irradiating a supersonic wave lightly and putting for a time. It carried at the tip of a plastics projectile and was air-dry.
[0020] Introductory processing of a gene was performed using the projectile which brought together the day [ of transplantation / 4th ] thing of a tobacco suspension culture cell BY-2nd (it receives from the Hokkaido University agricultural department) in ****** under sterile, put it on the sample base in equipment, and was air-dried under the following discharge conditions with the particle launcher. Discharge conditions are a degree of vacuum in the container which sets distance to a sample to 10cm, and stores a sample. The supply pressure of the nitrogen gas which adjusts to 100mmHg and is used for acceleration of a polyacetal projectile is about 28kg/cm2. It carried out (initial velocity per second about 200-250m). Moreover, that in which

the gene has not adhered to a magnetite particle as a control experiment was also fired on the same discharge conditions as a tobacco cultured cell at this time.
[0021] The gene was introduced into the tobacco cultured cell, and in order to evaluate that the function is discovered, assay using the 5-BUROMO-4-chloro-3-indolyl-glucuronide (X-Gluc) of an artificial substrate was performed. After firing a magnetite particle, the tobacco cultured cell which incubated at 25 degrees C for 24 hours was dipped in the phosphate buffer solution of X-Gluc the whole ****, and it incubated at 37 degrees C for 24 hours. Consequently, about about ten blue spots to on **** were observed.
[0022] The stereo microphotography of this spot is shown in drawing 2 . Abbreviation 100 or more blue tobacco cells (cell lump) were checked. This is because the beta-glucuronidase of an enzyme was formed into the tobacco cell of the work, X-Gluc of an artificial substrate was understood by the enzyme, as a result of introducing a gene, and in JIGOCHIN of blue coloring matter generated. An example which observed this tobacco cell with the little picking biological microscope is shown in drawing 3 . It was checked that the color is changing blue clearly as compared with the cell which has not discovered the color of the cell which the gene discovered. Moreover, when observed in the detail, it was observed that a nucleolus, an intracellular nuclear site, and intracellular cytoplasm are blue. In this experiment, installation of a gene and a manifestation were checked by about 1000 or more cells in all, and it succeeded in installation of the gene of a biological substance, and a manifestation for the first time by magnetite.
[0023] (Example 2) The magnetite particle which carried out the coat of the plasmid pBI221 of alternative concentration of the cell which carried out introductory processing of the magnetite particle to which the gene was made to adhere, and the separation above was fired, the tobacco cultured cell which performed introductory processing of a gene and incubated was suspended in optimum dose picking and a phosphate buffer solution, and it moved to the glass small specimen container, and experimented in the magnetic induction of this cell. The swing and the tobacco cell were uniformly distributed for the container ( drawing 4 ). Then, magnet (paper clip for stationery with a magnet) The photograph 2 minutes after making it stick to a container upper right portion is shown in drawing 5 R> 5. The tobacco cell of most in a container is carrying out accumulation to the right lateral upper part of a container so that clearly from drawing 5 , and only the cell containing the magnetite particle which has magnetism was alternatively separated and condensed by the MAG. Moreover, it experimented on the conditions same about the case where a magnetite particle and a tobacco cultured cell are only mixed as a control experiment. In this case, only the magnetite particle formed the assembly and the big and rough particle near the magnet promptly first. When concentration actuation with a magnet was performed about 3 times, only a magnetic magnetite particle moves and condenses, and the tobacco cell induced the MAG and stopped moving at all, although it was admitted that some tobacco cells were first influenced by motion of a particle, and it moved.
[0024] Thus, it was checked that only the tobacco cell which has magnetite can be alternatively condensed and divided into intracellular with the MAG.
(Example 3) Under the same conditions as the training aforementioned example of a tobacco culture cellular transformation object, the coat was carried out to the magnetite particle, having used only plasmid DNA as pBI121, and it introduced into the tobacco cultured cell similarly. The plasmid DNA used for the experiment this time makes the recombination into a vegetable chromosome cause.
[0025] The binary vector pBI121 is shown in drawing 6 . Plasmid DNA, beta-glucuronidase which can observe a manifestation part by dyeing as a reporter gene to pBI121 (GUS) It has a gene. This gene is a promotor who works with vegetation in order to enable a manifestation with vegetation. (promoter, P) Terminator (a polyadenylation part, poly-adenylation, pA) It is surrounded. NPTII is the promotor of a nopaline synthesis enzyme gene. (Pnos) Terminator of this gene (pAnos) GUS is a cauliflower mosaic virus. (CaMV) 35S promotor (pCaMV 35S) pAnos It is surrounded. Moreover, it has a kanamycin resistance gene as a marker gene.
[0026] The drug tolerance cell was screened by the culture medium which performs screening which carries out separation recovery of the cell which fired magnetite alternatively magnetically like the above-mentioned example, and contains antibiotics, such as JIENETISHIN, after that.

Under the present circumstances, both experiment which performed magnetic screening, and experiment which was not conducted were conducted, and screening effectiveness was compared. Consequently, that the efficiency of selection of a resistance callus is about about 10 to 20 times higher at least estimated [ the example of an experiment which does not perform magnetic screening ]. In addition, when the introductory gene was checked by the PCR method, existence of an introductory gene was accepted. Moreover, the band peculiar to an introductory gene was checked and the result of having performed Southern hybridization analysis was also considered that these cells are transformants.

[0027] As mentioned above, when a new magnetic screening procedure raised a transformant, it was shown that the process of screening of a time amount and time-consuming drug tolerance cell can be performed efficiently sharply.

[0028]

[Effect of the Invention] condensing and separating alternatively and specifically only the cell which the intracellular installation and its intracellular functional manifestation of a gene etc. of a biological substance of were attained using the particle which has magnetism, and the gene discovered or the physiological function discovered, and a cell lump and an organ according to this invention — things are made.

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TECHNICAL FIELD

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[Industrial Application] This invention relates to the approach the MAG condenses or separates alternatively the cell introduced by the approach and this approach of introducing biological substances, such as a gene and an enzyme, to a cell by the magnetic particle.

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PRIOR ART

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[0003] Moreover, the iron ultrafine particle (the minor axis of about 30nm, major axis of about 500nm) which has magnetism is encapsulated with synthetic macromolecule, and the support of the shape of an ultrafine particle which fixed the enzyme which can be guided magnetically is made by fixing an antibody and an enzyme in the outer surface.

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## EFFECT OF THE INVENTION

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TECHNICAL PROBLEM

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[Problem(s) to be Solved by the Invention] This invention aims at making intracellular installation and its intracellular functional manifestation of a gene etc. of a biological substance perform using the particle which has the magnetism of a physical property. Moreover, this invention aims at condensing alternatively and specifically and separating only the cell which the magnetic particle introduced into intracellular used magnetically the point which can be guided, for example, the gene discovered or the physiological function discovered, and a cell lump and an organ.
[0008] Plastics bead containing a conventional magnetic particle and a conventional magnetic particle (compounded magnetic microsphere) In all cases, such as an application, the magnetic particle was out of the cell, and it is impossible to current to put in a magnetic particle inside [ which has a hard cell wall like especially vegetation ] a cell, and it did not prove globally to this invention.

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## MEANS

[Means for Solving the Problem] It is the intracellular introductory approach of the biological substance characterized by this application the first invention firing into intracellular the magnetic particle which fixed the biological substance at high speed, and this application the second invention is alternative concentration and the separation method of the cell characterized by condensing or separating alternatively the cell into which the magnetic particle was introduced by this introductory approach with the MAG.
[0010] As a magnetic property in the magnetic particle used for this invention, if it is the magnetic substance, what kind of thing may be used, for example, ferromagnetism, a paramagnetism, and the superparamagnetism matter are used. As the quality of the material of this magnetic particle, for example A metal, a metallic oxide, nonmetal-metal complex, Although nature with ceramic complex and the above-mentioned matter, the organic magnetic substance containing a synthetic organic compound and its complex, the fluorescence matter, the optical responsibility matter, the optical magnetic functionality matter, the electron transport matter and the electrochemical functionality matter, the quantum-mechanical functionality matter, and semi-conducting material are mentioned From the passing speed by MAG, such as a cell which contains the particle in case magnetic induction separation and concentration are performed, being large The magnetic-substance particle which contains the compound particle of ferromagnetic elements, such as a ferromagnetic compound, for example, magnetite, other iron, cobalt, and nickel, and chromium, manganese, aluminum, an yttrium, a tellurium, a tungsten, titanium, etc. especially is desirable. Among these ferromagnetic compounds, toxicity is not shown and magnetite is [ in / to a living body / a stable point ] especially desirable.
[0011] As a particle size of this magnetic particle, a maximum grain size is one fifth of object cells. It is possible from grade to the size of an ultrafine particle, and is the mean particle diameter of 5nm - 100nm. An ultrafine particle or its floc can be used and it is the mean particle diameter of 10nm - several micrometers preferably. A magnetic particle is used. the consistency of this magnetic particle -- usually -- 1 - 21 g/cm3 -- desirable -- 3 - 8 g/cm3 it is . Therefore, magnetite (consistency : about 5g/cm 3), hematite (consistency: about 5 g/cm3), A cobalt ferric acid ghost (consistency : about 3g/cm 3), a barium ferrite (consistency : about 5g/cm 3), In addition, a consistency is 3 8g/cm. The carbon steel which is extent, tungsten steel, and KS steel, A consistency is 4 - 5 g/cm3. It is desirable to use the particle of the iron oxide which is extent, zinc, magnesium, manganese, or an iron compound (Ferroxcube) ingredient with nickel, and the particle of a rare earth cobalt magnet (consistency: about 8 g/cm3).
[0012] As for the gestalt of this magnetic particle, what has an angle in a cell wall or a cell membrane in a non-globular form in order to make it easy to break, penetrating and is desirable, and when compared with the same cross section, to a cell wall or a cell membrane, penetrating and since it is easy to overcome, moment of inertia is greatly needlelike, and a rod-like thing has a cross section more desirable than a spherical particle. In this invention, the biological substance which the above-mentioned magnetic particle is made to fix means physiological active substances, such as an animal, vegetation, protozoa, and a microorganism, an organella, a living thing particle, etc., for example, a gene, an enzyme, an antibody, protein, a pheromone, allomone, a mitochondrion, a virus, etc. are mentioned.

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[0015] The MAG can condense or separate alternatively the cell into which the magnetic particle was introduced as mentioned above. This concentration or separation can be performed as follows, for example. For example, the solution which distributed the cell is moved to transparence containers, such as a test tube, a magnet is stuck on the side attachment wall, and a cell is condensed. Then, only the cell containing a magnetic particle is separated by removing other solution parts with a pipet etc. This actuation is repeated several times if needed.
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## EXAMPLE

[Example] Hereafter, although an example explains this invention still more concretely, the range of this invention is not limited to the following examples.
(Example 1) as installation of the gene by magnetite, and a manifestation magnetism particle — magnetite (mean particle diameter 0.3 micrometers and consistency 5.2 g/cm3) (refer to drawing 1 ) using — party Kurgan — installation and its manifestation of a gene were performed using the particle launcher (Japanese-Patent-Application-No. 4-25626 equipment given in a number specification is used) by law.
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[0020] Introductory processing of a gene was performed using the projectile which brought together the day [ of transplantation / 4th ] thing of a tobacco suspension culture cell BY-2nd (it receives from the Hokkaido University agricultural department) in ****** under sterile, put it on the sample base in equipment, and was air-dried under the following discharge conditions with the particle launcher. Discharge conditions are a degree of vacuum in the container which sets distance to a sample to 10cm, and stores a sample. The supply pressure of the nitrogen gas which adjusts to 100mmHg and is used for acceleration of a polyacetal projectile is about 28kg/cm2. It carried out (initial velocity per second about 200-250m). Moreover, that in which the gene has not adhered to a magnetite particle as a control experiment was also fired on the same discharge conditions as a tobacco cultured cell at this time.
[0021] The gene was introduced into the tobacco cultured cell, and in order to evaluate that the function is discovered, assay using the 5-BUROMO-4-chloro-3-indolyl-glucuronide (X-Gluc) of an artificial substrate was performed. After firing a magnetite particle, the tobacco cultured cell which incubated at 25 degrees C for 24 hours was dipped in the phosphate buffer solution of X-Gluc the whole ****, and it incubated at 37 degrees C for 24 hours. Consequently, about about ten blue spots to on **** were observed.
[0022] The stereo microphotography of this spot is shown in drawing 2 . Abbreviation 100 or more blue tobacco cells (cell lump) were checked. This is because the beta-glucuronidase of an enzyme was formed into the tobacco cell of the work, X-Gluc of an artificial substrate was understood by the enzyme, as a result of introducing a gene, and in JIGOCHIN of blue coloring matter generated. An example which observed this tobacco cell with the little picking biological microscope is shown in drawing 3 . It was checked that the color is changing blue clearly as compared with the cell which has not discovered the color of the cell which the gene discovered. Moreover, when observed in the detail, it was observed that a nucleolus, an intracellular nuclear

site, and intracellular cytoplasm are blue. In this experiment, installation of a gene and a manifestation were checked by about 1000 or more cells in all, and it succeeded in installation of the gene of a biological substance, and a manifestation for the first time by magnetite.
[0023] (Example 2) The magnetite particle which carried out the coat of the plasmid pBI221 of alternative concentration of the cell which carried out introductory processing of the magnetite particle to which the gene was made to adhere, and the separation above was fired, the tobacco cultured cell which performed introductory processing of a gene and incubated was suspended in optimum dose picking and a phosphate buffer solution, and it moved to the glass small specimen container, and experimented in the magnetic induction of this cell. The swing and the tobacco cell were uniformly distributed for the container ( drawing 4 ). Then, magnet (paper clip for stationery with a magnet) The photograph 2 minutes after making it stick to a container upper right portion is shown in drawing 5 R> 5. The tobacco cell of most in a container is carrying out accumulation to the right lateral upper part of a container so that clearly from drawing 5 , and only the cell containing the magnetite particle which has magnetism was alternatively separated and condensed by the MAG. Moreover, it experimented on the conditions same about the case where a magnetite particle and a tobacco cultured cell are only mixed as a control experiment. In this case, only the magnetite particle formed the assembly and the big and rough particle near the magnet promptly first. When concentration actuation with a magnet was performed about 3 times, only a magnetic magnetite particle moves and condenses, and the tobacco cell induced the MAG and stopped moving at all, although it was admitted that some tobacco cells were first influenced by motion of a particle, and it moved.
[0024] Thus, it was checked that only the tobacco cell which has magnetite can be alternatively condensed and divided into intracellular with the MAG.
(Example 3) Under the same conditions as the training aforementioned example of a tobacco culture cellular transformation object, the coat was carried out to the magnetite particle, having used only plasmid DNA as pBI121, and it introduced into the tobacco cultured cell similarly. The plasmid DNA used for the experiment this time makes the recombination into a vegetable chromosome cause.
[0025] The binary vector pBI121 is shown in drawing 6 . Plasmid DNA, beta-glucuronidase which can observe a manifestation part by dyeing as a reporter gene to pBI121 (GUS) It has a gene. This gene is a promotor who works with vegetation in order to enable a manifestation with vegetation. (promoter, P) Terminator (a polyadenylation part, poly-adenylation, pA) It is surrounded. NPTII is the promotor of a nopaline synthesis enzyme gene. (Pnos) Terminator of this gene (pAnos) GUS is a cauliflower mosaic virus. (CaMV) 35S promotor (pCaMV 35S) pAnos It is surrounded. Moreover, it has a kanamycin resistance gene as a marker gene.
[0026] The drug tolerance cell was screened by the culture medium which performs screening which carries out separation recovery of the cell which fired magnetite alternatively magnetically like the above-mentioned example, and contains antibiotics, such as JIENETISHIN, after that. Under the present circumstances, both experiment which performed magnetic screening, and experiment which was not conducted were conducted, and screening effectiveness was compared. Consequently, that the efficiency of selection of a resistance callus is about about 10 to 20 times higher at least estimated [ the example of an experiment which does not perform magnetic screening ]. In addition, when the introductory gene was checked by the PCR method, existence of an introductory gene was accepted. Moreover, the band peculiar to an introductory gene was checked and the result of having performed Southern hybridization analysis was also considered that these cells are transformants.
[0027] As mentioned above, when a new magnetic screening procedure raised a transformant, it was shown that the process of screening of a time amount and time-consuming drug tolerance cell can be performed efficiently sharply.

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[Translation done.]

* NOTICES *



1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

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DESCRIPTION OF DRAWINGS

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[Brief Description of the Drawings]
[Drawing 1] It is the photograph in which the particulate structure of a magnetite particle is shown.
[Drawing 2] It is the photograph in which the gestalt of the living thing about a tobacco cell (cell lump) is shown.
[Drawing 3] It is the photograph in which the gestalt of the living thing at the time of observing the tobacco cell shown in drawing 2 with a little picking biological microscope is shown.
[Drawing 4] It is the photograph in which the gestalt of the living thing showing the condition of having distributed the tobacco cell uniformly is shown.
[Drawing 5] After distributing a tobacco cell uniformly, it is the photograph in which the gestalt of the living thing 2 minutes after sticking a magnet to a container upper right portion is shown.
[Drawing 6] It is drawing showing the binary vector pBI121.

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[Translation done.]

* NOTICES *



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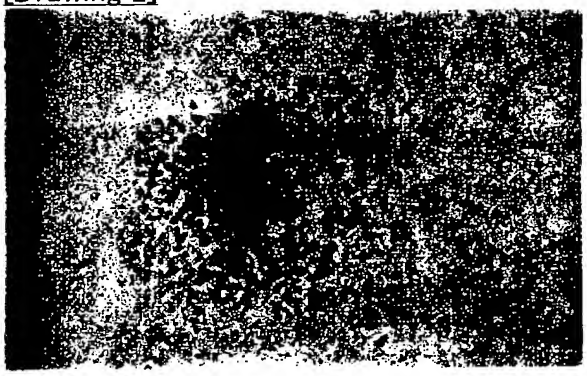
DRAWINGS

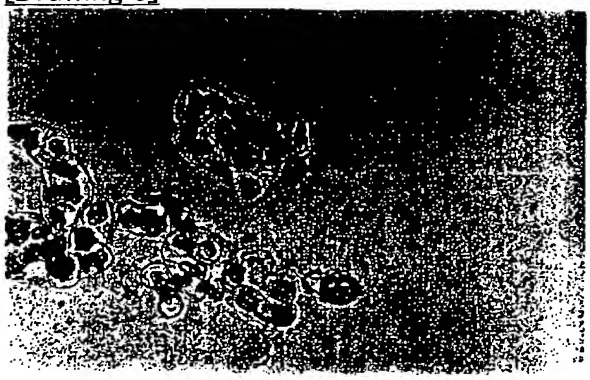
[Drawing 1]



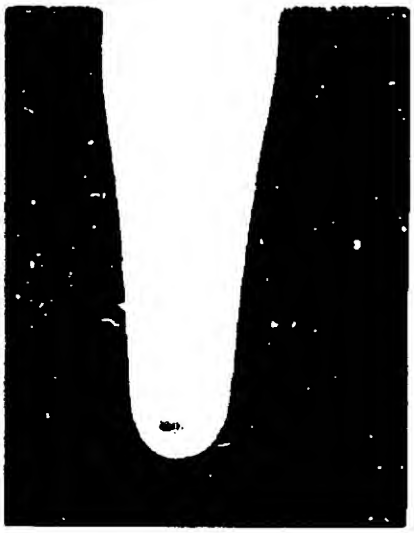
[Drawing 2]



[Drawing 3]



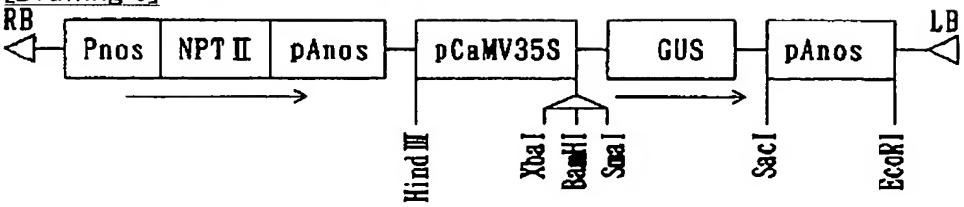
[Drawing 4]





[Drawing 5]

[Drawing 6]




バイナリーベクターpBI121

[Translation done.]

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(71)出願人　390014535
新技術事業団
東京都千代田区永田町２丁目５番２号

(71)出願人　592034168
角田　英男
北海道札幌市豊平区平岸１条１丁目４番12号

(72)発明者　角田　英男
北海道札幌市豊平区平岸１条１丁目４－12

(74)代理人　弁理士　平木　祐輔

(54)【発明の名称】　磁性微粒子による細胞への生体物質導入方法及び磁気による細胞の選択的濃縮・分離法

(57)【要約】

【構成】　生体物質を固定化した磁性微粒子を細胞内に高速で撃ち込むことを特徴とする生体物質の細胞内への導入方法、及び該導入方法により磁性微粒子が導入された細胞を磁気により選択的に濃縮又は分離することを特徴とする細胞の選択的濃縮・分離法。

【効果】　遺伝子等の生体物質の細胞内への導入とその機能発現が可能となり、また遺伝子が発現したり生理機能が発現した細胞や細胞塊、器官だけを選択的、特異的に濃縮、分離できる。

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【特許請求の範囲】

【請求項１】　生体物質を固定化した磁性微粒子を細胞内に高速で撃ち込むことを特徴とする生体物質の細胞内への導入方法。

【請求項２】　請求項１記載の導入方法により磁性微粒子が導入された細胞を磁気により選択的に濃縮又は分離することを特徴とする細胞の選択的濃縮・分離法。

【発明の詳細な説明】

【０００１】

【産業上の利用分野】本発明は、磁性微粒子により細胞へ遺伝子、酵素等の生体物質を導入する方法及び該方法により導入された細胞を磁気により選択的に濃縮又は分離する方法に関するものである。

【０００２】

【従来の技術】磁性微粒子について、P.J.Robinsonら(Biotechnol. Bioeng., 15, 603-606(1973))は、バイオリアクター用の酵素の固定化担体として、その回収操作が容易な点に着目して、磁性微粒子を酵素の固定化担体として用いる研究報告を行っている。この報告では、酸化鉄微粒子又はセルロースー酸化鉄複合体にα－キモトリプシン又はβ－ガラクトシダーゼを固定化し、完全混合型バイオリアクターに用いて、その磁性担体を磁気的に容易に凝集、分離できることが示されている。

【０００３】また、磁性を有する鉄の超微粒子（短径約30nm、長径約500nm)を合成高分子でカプセル化し、抗体や酵素をその外部表面に固定化することにより磁気で誘導可能な酵素などを固定化した超微粒子状の担体が作られている（「超微粒子　創造科学技術」林主税他編、三田出版会発行、231-235 頁）。ここでは、モデル酵素としてグルコースオキシダーゼを固定化した磁性超微粒子担体を用い小型のバイオリアクターにより反応を行わせた結果、単位体積当りの反応速度が高く、また超微粒子の担体を磁気で容易に濃縮、回収できることが示されている。

【０００４】更に、磁性粒子を分散させた高分子の微粒子として DYNABEADS（登録商標）が日本ダイナル（株）より輸入販売されている。この製品は、抗体その他の蛋白質や核酸などの磁気的分離を目的として酸化第二鉄（フェライト、$Fe_2O_3$）をポリスチレンビーズに分散させた微粒子（直径2.8 及び4.5 μm ）であり、この表面に抗体をコートしたり、ある種の蛋白質又はヌクレオチドなどを結合したビーズは磁石を併用することにより細胞の分離や精製を簡便な操作で速やかに行うことができる。

【０００５】しかし、上述した生体物質固定化磁性微粒子のうち、担体として磁性微粒子と高分子の複合体を用いるものは、撃ち込み用の微粒子としては密度が低く、衝突の衝撃力が弱いため不適当である。また、粒径が標的の細胞の大きさに比べて充分小さくない。従って、細胞壁を有する植物細胞内への導入は不可能であり、事実

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その前例はない。また、ガラスの細管を用いるマイクロインジェクション法では原理的に可能かもしれないが、１回で多数の細胞に導入することは困難である。

【０００６】また、J.C. Sanfordらによる米国特許第5,100,792 号明細書には遺伝子を微粒子担体に付着させ、高速で細胞内に導入し、その機能を発現させる方法（パーティクルガン法）が記載されており、ここでは微粒子の持つ衝突エネルギーを大きくするために、主に金やタングステンを用いている。この米国特許第5,100,792号明細書において、実施例で具体的に用いているのは直径４μm のタングステン微粒子であり、発明の詳細な説明中には、微粒子の粒径について約10nmないし数μm と記載され、高密度（約10～20ｇ／$cm^3$ ）のフェライトクリスタル、金、タングステン、その他の金属粒子、低密度（１～２ｇ／$cm^3$ ）のガラス、ポリスチレン、ラテックスビーズが記載されている。

【０００７】

【発明が解決しようとする課題】本発明は、物理的な性質の磁性を有する微粒子を用いて遺伝子などの生体物質の細胞内への導入とその機能発現を行わせることを目的とする。また、本発明は、細胞内に導入された磁性微粒子が磁気的に誘導可能である点を利用し、例えば遺伝子が発現したり生理機能が発現した細胞や細胞塊、器官だけを選択的、特異的に濃縮、分離することを目的とする。

【０００８】従来の磁性粒子や磁性粒子を含むプラスチックビーズ（複合された磁性ミクロスフェアー）の応用例などの場合は全て細胞外に磁性粒子があり、特に植物などのように硬い細胞壁を有する細胞の内側に磁性微粒子を入れることは現在まで不可能であり、本発明まで世界的に実証されていなかった。

【０００９】

【課題を解決するための手段】本願第一の発明は、生体物質を固定化した磁性微粒子を細胞内に高速で撃ち込むことを特徴とする生体物質の細胞内への導入方法であり、本願第二の発明は、該導入方法により磁性微粒子が導入された細胞を磁気により選択的に濃縮又は分離することを特徴とする細胞の選択的濃縮・分離法である。

【００１０】本発明に用いる磁性微粒子における磁気的性質としては、磁性体なら如何なるものでもよく、例えば強磁性、常磁性、超常磁性物質が用いられる。該磁性微粒子の材質としては、例えば金属、金属酸化物、非金属－金属複合体、セラミック複合体、上記物質との天然、合成有機化合物を含む有機磁性体、及びその複合体、蛍光性物質、光応答性物質、光磁気的機能性物質、電子伝達物質及び電気化学的機能性物質、量子力学的機能性物質、半導体物質が挙げられるが、磁気的な誘導分離や濃縮を行う際にその微粒子を含む細胞等の磁気による移動速度が大きいことから、特に強磁性化合物、例えばマグネタイト、その他鉄、コバルト、ニッケル等の強

磁性元素の化合物微粒子、またクロム、マンガン、アルミニウム、イットリウム、テルル、タングステン、チタン等を含む磁性体微粒子が好ましい。かかる強磁性化合物のうち、マグネタイトは生体に対して毒性を示さず、かつ安定である点において特に好ましいものである。

【００１１】該磁性微粒子の粒径としては、最大粒径が対象細胞の1/5 位から超微粒子のサイズまで可能であり、平均粒径５nm～100nm の超微粒子又はその凝集粒子を用いることができ、好ましくは平均粒径10nm～数μm の磁性微粒子が用いられる。該磁性微粒子の密度は、通常１～21ｇ／cm³ 、好ましくは３～８ｇ／cm³ である。従って、マグネタイト（密度：約５ｇ／cm³ ）、ヘマタイト（密度：約５ｇ／cm³ ）、コバルト鉄酸化物（密度：約３ｇ／cm³ ）、バリウムフェライト（密度：約５ｇ／cm³ ）、その他密度が８ｇ／cm³ 程度の炭素鋼、タングステン鋼、ＫＳ鋼や、密度が４～５ｇ／cm³ 程度の酸化鉄と亜鉛、マグネシウム、マンガン又はニッケルとの鉄化合物(Ferroxcube)材料の微粒子や希土類コバルト磁石（密度：約８ｇ／cm³ ）の微粒子を用いることが好ましい。

【００１２】該磁性微粒子の形態は、細胞壁や細胞膜に貫入、突破しやすくするため、非球形で角のあるものが好ましく、また同一断面積で比べると球状の微粒子よりも慣性モーメントが大きく細胞壁や細胞膜に貫入、突破しやすいため、針状で断面が棒状のものが好ましい。本発明において、上記磁性微粒子に固定化させる生体物質とは、動物、植物、原生動物、微生物等の生理活性物質、細胞内器官、生物微粒子等をいい、例えば遺伝子、酵素、抗体、蛋白質、フェロモン、アロモン、ミトコンドリア、ウィルス等が挙げられる。

【００１３】本発明において、固定化とは、生体物質を磁性微粒子に、主に物理的吸着やその他生物化学的親和力等によりその粒子に保持することをいう。上記生体物質の上記磁性微粒子への固定化は、例えば、該磁性微粒子の溶液と生体物質の緩衝溶液とを混合し、弾丸の先端に載せて風乾することにより行うことができる。

【００１４】本発明において対象となる細胞としては、動物細胞（ヒト細胞を含む）、植物細胞、その他の生物細胞、器官、組織等が挙げられる。本発明において、「高速で撃ち込む」とは、微粒子の初速が毎秒50～400m程度であることをいい、例えば、米国特許第5,100,792号明細書、特願平4-25626 号明細書等に記載のパーティクルガン法により行うことができる。

【００１５】上述のようにして磁性微粒子が導入された細胞は磁気により選択的に濃縮又は分離することができる。この濃縮又は分離は、例えば、次のようにして行うことができる。例えば、細胞を分散させた溶液を試験管等の透明容器に移し、その側壁に磁石を密着させて、細胞を濃縮する。その後、他の溶液部分をピペット等で除くことにより磁性微粒子が入っている細胞のみが分離される。必要に応じて、この操作を数回繰り返す。



【００１６】本発明は、遺伝子導入、組換え、組換え体の育成の効率化；生理効果（植物、動物）のある物質の導入と磁気誘導、選択分離；抗癌剤等の薬剤；遺伝子組換え細胞を用いるバイオリアクター（分析用－工業生産用）；磁気的遺伝子組換えのセンシング；細胞内の情報を磁気的手段とその他のあらゆる化学的、生化学的、物理的性質、方法を媒介として組み合わせた方法により外部から認識する手法等に応用することができる。

【００１７】

【実施例】以下、実施例により本発明を更に具体的に説明するが、本発明の範囲は以下の実施例に限定されるものではない。

（実施例１）　マグネタイトによる遺伝子の導入、発現磁性微粒子としてマグネタイト（平均粒径 0.3μm 、密度 5.2ｇ／cm³ ）（図１参照）を用い、パーティクルガン法により微粒子発射装置（特願平4-25626 号明細書記載の装置を使用）を用いて遺伝子の導入とその発現を行った。

【００１８】遺伝子にはプラスミドＤＮＡ，ｐＢＩ２２１（米国、クローンテック社製）を用いた。このプラスミドはβ－グルクロニダーゼ（ＧＵＳ）遺伝子を有し、これはカリフラワーモザイクウィルス（ＣａＭＶ）３５Ｓプロモーター（ｐＣａＭＶ３５Ｓ）とノパリン合成酵素遺伝子（ＮＯＳ）ターミネーター（pAnos)との間に接続されている。

【００１９】プラスミドＤＮＡのマグネタイト微粒子へのコーティングとプラスチック弾丸への付着操作は以下のように行った。50mgのマグネタイト微粒子を 100%エタノール１mlに懸濁させてある溶液を作り、この溶液とプラスミドＤＮＡのＴＥ緩衝溶液（ＤＮＡ濃度 200～500ng ／μl の間）と適量混ぜ、軽く超音波を照射して暫時静置した後、混合溶液を１～２μl プラスチック弾丸の先端に載せて風乾した。

【００２０】遺伝子の導入処理は微粒子発射装置により、タバコ懸濁培養細胞ＢＹ－２（北海道大学農学部より入手）の移植４日目のものを無菌下で濾紙上に集めて、それを装置内の試料台に載せ次の発射条件下で風乾した弾丸を用いて行った。発射条件は試料までの距離を10cmとし、試料を格納する容器内の真空度を 100mmHgに調節し、ポリアセタール弾丸の加速に用いる窒素ガスの供給圧力は約28kg／cm² とした（初速毎秒 200～250m程度）。また、この際、対照実験として遺伝子がマグネタイト微粒子へ付着してないものもタバコ培養細胞と同じ発射条件で撃ち込んだ。

【００２１】遺伝子がタバコ培養細胞に導入され、その機能が発現していることを評価するため人工基質の５－ブロモ－４－クロロ－３－インドリルーグルクロニド(X-Gluc)を用いたアッセイを行った。マグネタイト微粒子を撃ち込んだ後、25℃で24時間インキュベートしたタバ

コ培養細胞をX-Glucのリン酸緩衝液に濾紙ごと浸して37℃で24時間インキュベートした。この結果、濾紙のうえに青いスポットが約10箇所程観察された。

【００２２】このスポットの実体顕微鏡写真を図２に示す。約 100以上の青いタバコ細胞（細胞塊）が確認された。これは、遺伝子が導入された結果、その働きによりタバコ細胞中に酵素のβ－グルクロニダーゼが形成され、人工基質のX-Glucを酵素分解し、青色の色素のインジゴチンが生成したためである。このタバコ細胞を少量取り生物顕微鏡で観察した一例を図３に示す。遺伝子が発現した細胞の色は発現していない細胞と比較して明らかに色が青く変化しているのが確認された。また、詳細に観察すると、細胞内の核小体、核領域そして細胞質が青色であることが観察された。この実験では全部で約1000細胞以上に遺伝子の導入、発現が確認され、マグネタイトにより初めて生体物質の遺伝子の導入、発現に成功した。

【００２３】（実施例２）　遺伝子を付着させたマグネタイト微粒子の導入処理をした細胞の選択的濃縮、分離

上記の、プラスミドｐＢＩ２２１をコートしたマグネタイト微粒子を撃ち込み遺伝子の導入処理を行いインキュベートしたタバコ培養細胞を適量取り、リン酸緩衝液に懸濁し、ガラス製小型試料容器に移してこの細胞の磁気誘導の実験を行った。容器を振り、タバコ細胞を一様に分散させた（図４）。その後、磁石（文房具用磁石付紙クリップ）を容器右上部に密着させた２分後の写真を図５に示す。図５から明らかなように容器内の大部分のタバコ細胞が容器の右側面上部に濃集しており、磁性を有するマグネタイト微粒子を含む細胞だけが磁気により選択的に分離、濃縮された。また、対照実験として単にマグネタイト微粒子とタバコ培養細胞を混合させた場合について同じ条件で実験を行ってみた。この場合、まずマグネタイト微粒子だけが速やかに磁石の近くに集まり、粗大粒子を形成した。最初に一部のタバコ細胞が粒子の動きに影響されて移動するのが認められたが、磁石による濃縮操作を３回ほど行うと磁性のマグネタイト微粒子だけが移動、凝集してタバコ細胞は全く磁気に感応して動かなくなった。

【００２４】このように、細胞内にマグネタイトを有するタバコ細胞だけを選択的に磁気により濃縮、分離できることが確認された。

（実施例３）　タバコ培養細胞形質転換体の育成

前記実施例と同様の条件下で、プラスミドＤＮＡだけをｐＢＩ１２１としてマグネタイト微粒子にコートして同様にタバコ培養細胞に導入した。今回実験に用いたプラスミドＤＮＡは植物の染色体内への組換えを起こさせる。

【００２５】バイナリーベクターｐＢＩ１２１を図６に示す。プラスミドＤＮＡ、ｐＢＩ１２１にはレポーター遺伝子として、発現部位を染色により観察できるβ－グルクロニダーゼ（ＧＵＳ）遺伝子をもつ。この遺伝子は植物での発現を可能にするため植物で働くプロモーター（promoter, P)とターミネーター（ポリアデニル化部位、poly-adenylation, pA）に囲まれている。ＮＰＴＩＩは、ノパリン合成酵素遺伝子のプロモーター（Pnos）と同遺伝子のターミネーター（pAnos)に、ＧＵＳはカリフラワーモザイクウイルス（ＣａＭＶ）の３５Ｓプロモーター（pCaMV 35S)とpAnos に囲まれている。また、マーカー遺伝子としてカナマイシン耐性遺伝子を持っている。

【００２６】マグネタイトを撃ち込んだ細胞を上記の実施例のように磁気で選択的に分離回収するスクリーニングを行い、その後ジェネティシン等の抗生物質を含む培地で薬剤耐性細胞のスクリーニングを行った。この際、磁気的スクリーニングを行った実験と行わなかった実験の両者を行ってスクリーニング効率を比較した。この結果、磁気的なスクリーニングを行わない実験例と比較して、例えば少なくとも約10～20倍位耐性カルスの選抜効率が高いことが見積もられた。なお、ＰＣＲ法により導入遺伝子の確認を行ったところ、導入遺伝子の存在が認められた。また、サザンハイブリダイゼーション分析を行った結果も導入遺伝子特有のバンドが確認され、これらの細胞は形質転換体であると考えられた。

【００２７】以上のように、新しい磁気的スクリーニング法は、形質転換体を育成する時に時間と手間がかかる薬剤耐性細胞のスクリーニングのプロセスが大幅に効率的に行えることが示された。

【００２８】

【発明の効果】本発明によれば、磁性を有する微粒子を用いて遺伝子等の生体物質の細胞内への導入とその機能発現が可能となり、また遺伝子が発現したり生理機能が発現した細胞や細胞塊、器官だけを選択的、特異的に濃縮、分離することことができる。

【図面の簡単な説明】

【図１】マグネタイト微粒子の粒子構造を示す写真である。

【図２】タバコ細胞（細胞塊）についての生物の形態を示す写真である。

【図３】図２に示すタバコ細胞を少量取り生物顕微鏡で観察した場合の生物の形態を示す写真である。

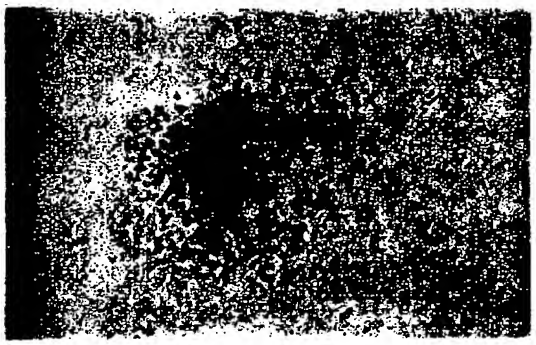
【図４】タバコ細胞を一様に分散させた状態を表す生物の形態を示す写真である。

【図５】タバコ細胞を一様に分散させた後、磁石を容器右上部に密着させた２分後における生物の形態を示す写真である。

【図６】バイナリーベクターｐＢＩ１２１を示す図である。

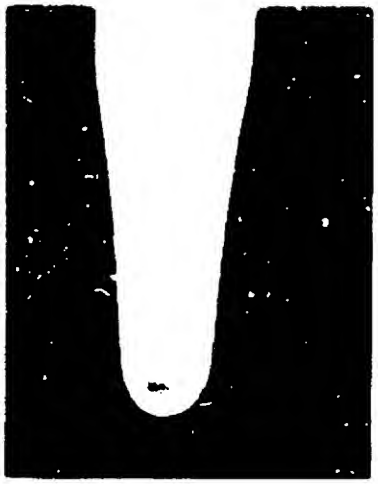
【図１】

【図２】



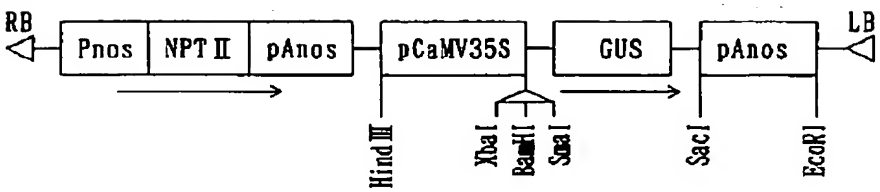
【図３】

【図４】



【図５】

【図６】

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